Interpretative Summary: Effect of processing on polyamine content and bioactive peptides released after in vitro gastrointestinal digestion of infant formulas. Gómez-Gallego, *et al*.

This study examined the influence of processing on polyamines and peptide release after the digestion of a commercial infant formula designed for child feeding during first months of life. As compositional dissimilarity between human milk and infant formula in polyamines and proteins, could be responsible for some of the differences in health reported between breast-fed and formula-fed children, the changes observed along processing must be taken into consideration because they may have a great impact on infant nutrition and development.

Running head: **EFFECT OF PROCESSING ON POLYAMINES AND PEPTIDES IN INFANT FORMULAS**

Effect of processing on polyamine content and bioactive peptides released after *in vitro* gastrointestinal digestion of infant formulas

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ABSTRACT

This study examined the influence of processing on polyamines and peptide release after the digestion of a commercial infant formula designed for children during first months of life. Polyamine oxidase activity was not suppressed during the manufacturing process, which implicate that polyamine concentrations were reduced over time and during infant formula self-life. In gel electrophoresis, in vitro gastrointestinal digestion of samples with reduced amount of enzymes and time of digestion shows an increase in protein digestibility, reflected in the increase in non-protein nitrogen after digestion and the disappearance of β-lactoglobulin and α-lactalbumin bands in gel electrophoresis. Depending on the sample, between 22 and 87 peptides were identified after gastrointestinal digestion. A peptide from β-casein f(98–105) with the sequence VKEAMAPK and antioxidant activity appeared in all of the samples. Other peptides with antioxidant, immunomodulatory, and antimicrobial activities were frequently found, which could have an impact on infant health. The present study confirms that the infant formula manufacturing process determines the polyamine content and peptidic profile after digestion of the infant formula. As compositional dissimilarity between human milk and infant formula in polyamines and proteins, could be responsible for some of the differences in health reported between breast-fed and formula-fed children, these changes must be taken into consideration because they may have a great impact on infant nutrition and development.

KEYWORDS: infant formula; polyamines; peptides, simulated gastrointestinal digestion; mass spectrometry.

INTRODUCTION

Breast milk has a complex composition of nutrients and bioactive components designed to fulfill the needs of the growing infant. In recent years, the infant food industry has made an effort to develop infant formulas that are more similar to human milk to improve the nutrition of infants who are not breastfeeding.

Protective compounds, such as cytokines, oligosaccharides, and even microbes, in breast milk provide the newborn with the means to adapt to the environment (Gueimonde et al., 2007; Newburg and Walker ,2007). Among the bioactive compounds found in breast milk are polyamines, such as, spermidine, spermine, and putrescine, as well as bioactive peptides released during milk protein digestion. Polyamines have a positive effect on the development of gastrointestinal tract (Larqué et al., 2007) and immune system (Pérez-Cano et al., 2010; Gómez-Gallego et al., 2014 b). The levels and effects of these compounds in infant formulas compared with human milk are of special interest, as their concentrations are lower than in human milk (Buts et al., 1995). Moreover, dietary proteins are a source of biologically active peptides that are inactive within the sequence of parent protein and can be released during gastrointestinal digestion or food processing. Once bioactive peptides are liberated, they may act as regulatory compounds. Bioactive peptides are widely distributed among milk protein sequences (Clare et al., 2000) which can be released during digestion in vivo. However, changes that take place in protein structure during the manufacturing of infant formulas can influence protein digestion and peptide liberation (Korhonen et al., 1998).

Technological processes used in food manufacturing affect the functional, nutritional, and biological properties of food components. Depending on the intensity of the heat

48 treatment, the nutritive value of proteins can be affected in a positive or negative way
49 (Korhonen et al., 1998) and, by extension, can affect other related compounds.

50 The structure of milk is greatly altered depending on the various mechanical and
51 thermal steps of the processing chain (Michalski and Januel, 2006). Heating and
52 homogenization are the most common and most widely used method capable of
53 modifying proteins during infant formula manufacturing. Among the different physical
54 and chemical changes, a great deal of attention has been focussed on the covalent
55 interaction protein/carbohydrate via the Maillard reaction. During this reaction, the
56 conjugation of a reducing carbohydrate to the 3-amino group of lysine occurs
57 spontaneously under heating conditions (Corzo-Martínez et al., 2012). Some studies
58 have shown contradicting effects regarding the Maillard reaction. On one hand,
59 glycation can lead structural changes, which could generate new enzymatic cleavage
60 sites (Corzo-Martínez et al., 2012, Joubran et al., 2015) modulating protein digestibility
61 and peptides release after digestion. On the other hand, conjugation could limit
62 enzymatic accessibility through steric hindrance (Joubran et al., 2015). These two
63 counteracting effect could explain the different digestive patterns reported for other
64 authors. Moreover, heat treatment changes the impact of homogenization on milk
65 structure (Michalski and Januel, 2006). The main effect of homogenization on soluble
66 milk components is the disruption of casein micelles in micellar form or as fragments.
67 Homogenization seems to improve milk digestibility, however, heat treatment changes
68 the impact of homogenization on milk structure and has been reported that infants digest
69 better native human milk fat globules than homogenized droplets from infant formula
70 (Michalski and Januel, 2006). As concluded by Michalski and Januel (2006), the
71 structural consequences in milk proteins seem to depend on the sequence of the

homogenization and heat treatments, but they are rather controversial due to the various treatments applied and to the different procedures used in the food industry.

The aims of this study were to evaluate how formula processing influences polyamine content and peptide release after digestion. Furthermore, the behavior of the polyamine oxidase activity of the milk and the digestibility of proteins was studied. The results of this work could be a preliminary step to improving infant formula composition, which could promote better health status of children fed with infant formulas during the first months of life.

MATERIALS AND METHODS

Samples. The infant formula samples used in this study were supplied by Hero España S.A. (Alcantarilla, Spain) at different representative stages along the manufacturing process. Figure 1 shows the flow diagram of the infant formula manufacturing process and the steps at which the samples were taken. The samples were: F1) cow milk used as raw material in infant formula processing; F2) cow milk after skimming and the first thermal treatment; F3) concentrated milk after the second thermal treatment; F4) concentrated infant formula after the last thermal treatment; F5) the infant formula final product; and S) milk whey used as an ingredient. Whey was demineralised sweet whey from cow's milk, which a content of lactose around 70%, added as ingredient to increase milk serum proteins in the final product to 60% of total proteins.

One kg of powdered infant formula (F5) and 500 ml aliquots of liquid samples (F1, F2, F3, F4, and S) were taken from five different batches separately. Liquid samples were lyophilized, and all of the samples were preserved at -20 °C until analysis.

Determination of moisture and nitrogen. Moisture (method 964.22) along the infant formula manufacturing process and nitrogen (**N**) content using the micro-Kjeldahl procedure (method 955.04) were determined in the samples and digestions using official AOAC methods (AOAC 1990). Protein calculations were made using 6.25 as the conversion factor. Non-protein nitrogen (**NPN**) in the samples and digestions was estimated using the micro-Kjeldahl method after dissolving 20 g of the sample in 100 ml of 15% trichloroacetic acid (**TCA**) for milk protein precipitation and filtration.

Analysis of polyamines. A high-performance liquid chromatography (**HPLC**) method using a diode array detector was used. The HPLC system consisted of a Waters 2690 system connected to a Waters 910 detector. The analytical column was a Spherisorb® 5.0-µm ODS2, 4.6 mm × 150 mm (Waters, Milford, CT). Detection was performed at 254 nm.

The samples were diluted ten times with a solution of TCA as described by Nishibori et al. (2007), but the concentration was adjusted to 15% for milk protein precipitation. The samples were homogenized using gentle agitation for 30 min (Pollack et al., 1992). After centrifugation at 13000 × g for 15 min at 4 °C, the supernatants were filtered using 0.45-µm membrane filters (Whatman, Brentford, England) and dansylated by adapting the method described by Buts et al. (1995). Clear supernatant (1 ml) was basified by adding 250 µl of saturated sodium carbonate and 1 ml of dansyl chloride solution (10-mg/ml acetone; Fluka, Steinheim, Germany).

After incubation, 200 µl of L-proline solution (Scharlau, Barcelona, Spain) was added to clean the excess dansyl chloride (Escribano, 1990). Extraction of dansyl derivatives

was performed twice with cyclohexane, and organic phases were collected and evaporated in nitrogen air flow. Residues of dansyl derivatives were dissolved in 1-ml acetonitrile (Merck, Darmstadt, Germany) and filtered with 0.45-µm HPLC filters (Upchurch Scientific, Oak Harbor, WA). Aliquots of 20 µl were injected in the HPLC. Samples were run for 30 min according to a linear gradient method including two mobile phases: water and acetonitrile (Table 1).

Each sample was analyzed in duplicate. Quantification of the polyamine concentration was made by comparing the integrated surface areas of peaks with areas of dansylated polyamines of known concentration on a standard curve. To avoid mistakes due to loss of dansylated polyamines during manipulation, 1,3-diaminopropane (Aldrich, Steinheim, Germany) was used as the internal standard (Tovar, 2002). Polyamine concentrations were expressed in parts per million.

Analysis of polyamine oxidase activity. The polyamine oxidase (**PAO**) activity assay was performed using the method described by Suzuki et al. (1984), in which hydrogen peroxide formed by polyamine oxidase is measured fluorometrically by converting homovanillic acid to a highly fluorescent compound by peroxidase, adding pargyline and semicarbazide for monoamine oxidase and diamine oxidase inhibition, respectively. A multi-mode microplate reader (Synergy 2; Bio-Tek, Winooski, VT); fluorescence filters with excitation and emission wavelengths of 360 and 460 nm respectively; and 96 well black, flat bottom microplates were used to carry out this assay. Each sample was analyzed in triplicate. Results were expressed as µmoles H_2O_2/mg dry extract/30 min.

In vitro gastrointestinal digestion of samples. The hydrolysis procedure was based on the method described by Miller et al. (1981) and comprised the simulation of gastric and intestinal digestion of the samples by *in vitro* enzymatic treatment. The modifications introduced by different authors (Bosscher et al., 2001; Jovani et al., 2001; Hernández-Ledesma et al., 2007; Frontela et al., 2008; Bourlieu et al., 2014) reduced the concentrations of digestive enzymes and the time of digestion, to simulate gastrointestinal conditions during lactation. In the first step, 3.9 g of the dry extract of the sample were dissolved in 10 mL of Milli-Q water obtained using a Milli-Q water purification system (Millipore, Molsheim, France). Then, the pH was adjusted to 4.0 with 1M HCl, and the samples were hydrolyzed with pepsin (from porcine gastric mucosa; Sigma, St. Louis, MO) at a ratio of 10.7 mg of pepsin/g of sample, for 30 min at 37 °C. Directly afterward, the pH of the digested sample was raised to 5.0 using sodium hydrogen carbonate 1M, and the samples were hydrolyzed with pancreatin (from porcine pancreas; Sigma) and bile salts (Fluka), at a ratio of 2-mg pancreatin/g of the sample and 6.25-g bile salts/g of pancreatin. Intestinal digestion with pancreatin was performed at 37 °C for 90 min. Digestion was carried out in a thermally controlled incubator under constant stirring. The reaction was stopped, the sample was put on ice, and the pH was adjusted to 7.2 with sodium hydrogen carbonate 1M. The enzymes were inactivated by heating at 95 °C for 15 min in a water bath, followed by cooling to room temperature. A fraction of the digestions were run in gel electrophoresis to check for the presence of proteins and changes in protein composition. Digestibility and RP-HPLC-MS/MS analyses were performed to evaluate influence of the processing in peptide release after digestion. In vitro gastric and intestinal digestions and subsequent analyses were carried out at least in duplicate.

166 Protein digestibility was calculated as the increase in NPN following gastrointestinal
167 digestion, as described by Rudloff and Lönnerdal (1992). Nitrogen content of fractions
168 was determined as described previously employing Kjeldahl procedure.

169 **Gel electrophoresis.** To investigate the protein composition of milk fractions during
170 digestion, the molecular weights of the proteins were analyzed by gel electrophoresis in
171 sodium dodecyl sulfate (**SDS**). The analysis was conducted using a PhastSystem™
172 electrophoresis system (Pharmacia, Uppsala, Sweden) as described by Jiménez-Saiz et
173 al. (2011), with precast homogenous gels 20% (GE Healthcare, New York, NY) and
174 PhastGel™ SDS buffer strips (Amersham Biosciences Corp., Uppsala, Sweden),
175 following the manufacturer's instructions for the electrophoretic and Coomassie staining
176 conditions. The samples were dissolved in 10-mM Tris-HCl buffer, pH 8.0, containing
177 2.5% SDS and 10 mM EDTA, and heated at 95 °C. They were then analyzed in the
178 presence of 5% 2-β-mercaptoethanol.

179

180 **Analysis by on-line RP-HPLC-MS/MS.** A water-soluble hydrolysate extract was
181 obtained by centrifugation at 20000 × g for 30 min at 5 °C and by filtration through a
182 Whatman no. 41 filter (GE Healthcare Bio-Sciences, Pittsburgh, PA). The water-soluble
183 extract was subjected to ultrafiltration through a hydrophilic 1000-Da cutoff membrane
184 (Amicon Inc., Beverly, MA).

185 The hydrolysates were injected into an HPLC system (Agilent, Santa Clara, CA), which
186 was connected on-line to an Esquire-LC quadrupole ion trap instrument (Bruker
187 Daltonik, Bremen, Germany), according to the method of Hernández-Ledesma et al.
188 (2004). The column used in these experiments was a 250-mm × 4.6-mm Discovery BIO
189 Wide Pore C18 column (Bio-Rad, Richmond, CA). A Nova-Pak® C18 guard column

20 mm × 3.9 × 4 µm (Waters Corp., Milford, MA) was used to protect the analytical column. The injection volume was 50 µl. Solvent A was a mixture of water and trifluoroacetic acid (1000:0.37, v/v), and solvent B contained acetonitrile and trifluoroacetic acid (1000:0.27, v/v). Peptides were eluted with a linear gradient of 0–45% solvent B over 60 min at a flow rate of 0.8 ml/min. The signal threshold to perform auto MS(n) analyses was 1,000, and the precursor ions were isolated within a range of 4.0 m/z and fragmented with a voltage ramp going from 0.35–1.4 V. The m/z spectral data were processed using Data Analysis 3.0 (Bruker Daltonik) and transformed to spectra representing mass values. MS(n) spectra were processed in BioTools 2.1 (Bruker Daltonik) to perform peptide sequencing.

Statistical analysis. Statistical analyses were conducted using ANOVA and relevant post-hoc tests with SPSS 15.0 software. Differences between means were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Impact of processing on polyamine content and polyamine oxidase activity of infant formulas. The concentrations of the polyamines detected in the analyzed samples varied over a wide range, probably reflecting differences in the stages of the process from where the samples were taken, the original raw milk of the batch, and the PAO activity of the sample. The results are summarized in Table 2. Overall, polyamine content was lower in the first steps of the processing than in the last steps or final product. This could be due to the PAO activity in these samples (Table 3) influenced for whey addition before the last concentration stage. With respect to PAO activity of the

raw milk, the final infant formula retained more than 60% of the enzymatic activity, which was not inactivated due to the high resistance of the enzyme. This persistent PAO activity changes polyamine concentrations over time and during infant formula reconstitution.

As reported in previous studies (Buts et al. 2005; Pollack et al. 1992; Romain et al. 1992), the polyamine content in infant formula is around ten times less than that of human milk. In our samples, if the formula was reconstituted according to the manufacturer's instructions, the final concentrations of putrescine, spermidine, and spermine would be 0.317, 0.075, and 0.061 ppm, respectively. These amounts were similar to those reported by Romain et al. (1992), with higher levels for putrescine. Previous studies (Buts et al., 2005; Pollack et al., 1992; Romain et al., 1992, Dorhout et al., 1996) established a great variation in milk composition from one mother to another, and even between the left and right breasts (Buts et al., 2005), but the average concentrations of polyamines were around 0.058, 0.580, and 0.825 ppm for putrescine, spermidine, and spermine, respectively. Thus, the concentrations of spermidine and spermine, the most active polyamines (Larqué et al., 2007) in manufactured formulas, were much lower than in human milk, although the concentration of putrescine was higher than in breast milk. This finding reveals the lack of an important functional compound in infant formulas compared with breast milk, and due to the role of polyamines on immune system and intestinal microbiota development we recently reported (Gómez-Gallego et al., 2014 a, Gómez-Gallego et al., 2014 b) the development of infant formulas with a high but safe polyamine content must be take into consideration.

Impact of processing on digestibility of milk proteins in infant formulas. The protein composition of fractions was further characterized by gel electrophoresis. We found that the processing of infant formulas had an influence on the protein structure. The comparison between raw milk (F1) and infant formula (F5) shows that processing alters bands on SDS-gels (Figure 2; lanes 1 and 2, versus 5 and 6). As reported by other authors (Barbé et al., 2013) heat treatment induced susceptibility to hydrolysis, thereby facilitating protein digestion, which was reflected in band comparison after gastric and gastrointestinal digestion (Figure 2; lanes 3 and 4, versus 7 and 8). It is mainly evident in the β-lactoglobulin and α-lactalbumin bands, which persist in raw milk after gastrointestinal digestion and practically disappear during digestion in infant formula samples. In raw milk, β-lactoglobulin and α-lactalbumin remain undegraded due to the partial resistance to pepsin and pancreatin in the soft acidic conditions of infant in vitro digestion (Korhonen et al., 1998). In the whey samples, proteins seemed to have relatively high resistance to the conditions of digestion, which was reflected in the persistence of the bands for β-lactoglobulin and α-lactalbumin, even after *in vitro* digestion (Fig 2; lane 12). These results are according with data published with other authors, demonstrating that milk proteins persisted longer under infant digestion condition (Joubran et al., 2015), potentially generating bioactive peptides.

Digestibility was related with the increase in NPN that resulted from enzymatic digestion is shown in Table 4. Protein digestibility, related with the increase in NPN was lower in whey (S), that supported the results confirmed by gel electrophoresis. It appeared that manufacturing process exerted whey protein denaturation, which facilitated its digestion. At the end of the F5 sample digestion estimated in vitro digestibility of the F5 sample increased from 17’9% of the F1 sample to 44.9%. As reported by Rudloff and Lönerdal (1992), this low digestibility could be due to the soft

gastrointestinal digestion conditions during the first months of life as well as lipid-protein or peptide interactions. But our results were even lower than those reported by other authors.

It is important to consider that in vitro digestibility is only an approximation of the physiological process and does not take into account other characteristics of real digestion, such as gastric emptying, intestinal fluid, intestinal motility, and mucosal enzymes. However, it could be considered a warning regarding real protein digestion during lactation (Binaghi et al., 2002).

Identified peptides after gastrointestinal digestion. The ultrafiltration permeates of the digested samples were obtained and subjected to tandem MS for peptide identification. A total of 49 protein fragments could be identified for raw milk (F1) (Table 1S, available online), 29 for skimmed milk (F2) (Table 2S, available online), 22 for concentrated milk (F3) (Table 3S, available online), 23 for milk serum (S) (Table 4S, available online), 55 for concentrated manufactured formula (F4) (Table 5S, available online), and 87 for infant formula (F5) (Table 6S, available online).

There is growing evidence that the peptide sequences released during digestion can vary depending on the manufacturing process of infant formulas. As suggested by other authors (Chang et al., 1981), digestion experiments with infant formulas may provide information regarding the susceptibility of proteins to gastrointestinal conditions, hydrolysis, and bioactive peptide release in infant formulas.

The comparison of the majority peptide profiles in the 1000 Da permeates showed that changes in the profile of peptides released and identified was greatly dependent on the processing stage of the infant formula. Our results showed that the percentage increases of peptides originating from whey proteins during processing—from 33.3% in raw milk

(F1) to 49.9% in final infant formula (F5)—may have been due to the addition of whey (S) as an ingredient.

With the exception of the whey samples (S), a peptide from β-casein f(98-105) with the sequence VKEAMAPK and antioxidant activity (Korhonen and Pihlanto-Leppäla, 2007) appeared in all of the samples. Other abundant peptides in the samples were ETYG, ELLK, STAV, LPDT, AVVKK, ATAST, IIAEK, EAMAPK, RELEE, RYLG, LRLK, LRLKK, LKKI, VQVTSTAV, YLGY, YSLA, YLGYL, VEGPKLV, and HAQ. Among these peptides, the αs1-casein fragment f(91-94), with the sequence YLGY, had a structure potentially able to act as an opioid receptor ligand. This peptide is a fragment of α-casomorphin (Clare and Swaisgood, 2000), which maintains an amino-terminal tyrosine for essential opioid activity (Chang et al., 1981) and an aromatic amino acid in the third or fourth position that favors peptide union to opioid receptor (Kohmura et al., 1990). Further studies are necessary to confirm the potential opioid activity of this fragment, because it may have an important role in sleeping pattern and the development and function of the gastrointestinal tract in infants depending on its opioid agonistic or antagonistic activity (Wada and Lönnerdal, 2014).

Additionally, some of the peptide sequences found in F1, F2 and F5 samples have been found to exert angiotensin I-converting enzyme (ACE)-inhibitory activities. Their sequences were also described in human milk proteins and can be expected that breast-fed infants as well as infants fed with milk-based formulas will obtain ACE-inhibitory peptides, which may play a role in cardiovascular health. It has been proposed than the immaturity related higher serum ACE activity in early life could program cardiovascular disease later in life but that ACE-inhibitory peptides present in milk might improve this negative effect (Wada and Lönnerdal, 2014). So their presence in

311 formulas and their activity compared with breast milk should be an object of further
312 studies.

313 Moreover, in the profile of peptides released after gastrointestinal digestion of the final
314 manufactured formula (Table 6S), peptides with antioxidant (HIQKEDVPSER,
315 VKEAMAPK) (Korhonen and Pihlanto-Leppäla, 2007; Gupta et al., 2010),
316 antimicrobial activity (IPAVFK) (Pellegrini et al., 2001), ACE-inhibitory peptides
317 (FRQF, PLW, RPK, RVY, IIAEK) (Contreras et al., 2009, Nagaoka et al., 2001;
318 Shahidi and Zhong, 2008; Matsufuji et al., 1994; Maruyama et al., 1987), a peptide
319 modulator of non classical cadherins functions (QUINNK) (Blaschuk et al., 1999) and a
320 cathepsin B inhibitor (PFPGPI) (Lee and Lee, 2000) were found. It is important to
321 highlight that our results shows some fragments originating from β-lactoglobulin, a
322 protein absent in human milk. Two of this peptides—RVY and IPAVFK—had reported
323 to exert ACE-inhibitory and antibacterial activity respectively. Additional studies are
324 necessary to determine whether these peptides have similar functional properties to
325 human milk, defined for a different peptide profile (Wada and Lönnerdal, 2014). In
326 addition, the fragments of many previously characterized bioactive peptides were found
327 after infant formula (F5) gastrointestinal digestion. Some of these fragments may have
328 preserved similar bioactivity of the origin sequence and must be taken in consideration
329 for further studies.

330 The differences observed on the peptidic profile in each step of the processing could be
331 related with the sequence and conditions of heat treatments along the manufacturing
332 process. As Maillard conjugation could modulate the functionality and digestibility of
333 milk proteins, the knowledge of how processing under well controlled conditions
334 change the peptides release after gastrointestinal digestion could be useful for

335 improving functionality through the generation of bioactive peptides (Corzo-Martínez
336 et. al 2012; Joubran et al., 2015).

337 Antimicrobial and immunomodulatory peptides, together with polyamines, may be
338 responsible for the different susceptibility to some diseases between formula-fed and
339 breast-fed infants. ACE-inhibitory peptides may play a role in cardiovascular health
340 (Wada and Lönnerdal, 2014), and antioxidant peptides may protect infants from
341 oxidative stress-associated diseases, such as necrotizing enterocolitis (Okur et al.,
342 1995).

343

344 CONCLUSIONS

345 The present study confirms that the infant formula manufacturing process determines,
346 together with raw milk composition, the polyamine content and peptidic profile after
347 digestion of the infant formula. Therefore, compositional dissimilarity between human
348 milk and infant formula in polyamines and proteins, and the functionality of these
349 proteins and their peptides, could be responsible for some of the differences in health
350 reported between breast-fed and formula-fed children. These changes must be taken
351 into consideration because they may have a great impact on infant nutrition and
352 development.

353 Further studies are important to address the effects of manufacturing process in protein
354 digestibility, peptides profile and polyamine content during formulation of infant
355 formulas for the potential implication in human health.

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357

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595 TABLES

Table 1. Gradient of acetonitrile:water employed by dansylated polyamines separation.

Time (min)	% Acetonitrile	% Water
0	60	40
5	60	40
15	70	30
20	95	5
25	98	2
26	98	2
26.1	61	39
30	60	40

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Table 2. Polyamine concentration (mg/Kg) in dry samples along manufacturing process

Sample	Putrescine		Spermidine		Spermine	
F1	**0.650**^{a}	0.576-0.749	**0.057**^{a}	0-0.124	**0.065**^{a}	0-0.140
F2	**1.047**^{a,b}	0.680-1.380	**0.126**^{a}	0.116-0.151	**0.125**^{a,b}	0.124-0.129
F3	**1.858**^{a,b,c}	0.170-3.950	**0.967**^{b,c}	0.038-1.779	**0.469**^{a,b,c}	0-1.084
F4	**2.824**^{c}	1.369-7.757	**1.118**^{c}	0.661-2.009	**0.756**^{b,c}	0.376-1.367
F5	**2.441**^{b,c}	1.339-3.492	**0.576**^{a,b}	0.271-0.983	**0.466**^{a,b,c}	0.137-1.210
S	**3.109**^{c}	1.670-5.610	**0.406**^{a}	0-0.761	**0.948**^{c}	0-1.935

F1) cow milk; F2) cow milk after skimming and the first thermal treatment; F3) concentrated milk after the second thermal treatment; F4) concentrated infant formula after the last thermal treatment; F5) the infant formula final product; and S) milk whey used as an ingredient.
Each value represents the mean (bold) and the range

Differents letters in the same column indicates statistical significant differences at the level of $p<0.001$

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Table 3. PAO activity (µmoles H_2O_2/mg dry extract/30min) in samples along manufacturing process and evolution of the percentaje of relative PAO activity comparing with raw milk

Sample	PAO actvity (mean ± SD)		Relative PAO activity (%)
F1	**21.59**[a]	± 2.31	**100.00**
F2	**25.27**[a]	± 0.72	**117.04**
F3	**11.94**[b]	± 1.43	**55.30**
F4	**11.98**[b]	± 3.58	**55.49**
F5	**14.48**[b]	± 0.45	**69.38**
S	**15.44**[b]	± 3.23	**71.51**

F1) cow milk; F2) cow milk after skimming and the first thermal treatment; F3) concentrated milk after the second thermal treatment; F4) concentrated infant formula after the last thermal treatment; F5) the infant formula final product; and S) milk whey used as an ingredient.
Differents letters in the same column indicates statistical significant differences at the level of $p<0.001$

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Table 4. Digestibility of proteins in samples along infant formula manufacturig process.

	Moisture (%)	Protein in dry extract (mg/g)	NPN before digestion in dry extract (mg/g)	NPN before digestion in dry extract (mg/g)	Digestibility (%)
F1	88.17	26.84	0.50 ± 0.21	1.27 ± 0.21	17.89 ± 5.2[b]
F2	91.18	37.45	0.35 ± 0.11	1.23 ± 0.26	14.87 ± 4.64[b]
F3	60.38	10.89	0.18 ± 0.12	0.97 ± 0.20	45.64 ± 11.80[a]
F4	61.11	11.23	0.24 ± 0.03	1.03 ± 0.21	44.99 ± 11.57[a]
F5	1.69	10.57	0.19 ± 0.02	0.95 ± 0.20	45.19 ± 11.29[a]
S	79.14	69.72	0.90 ± 0.09	1.45 ± 0.08	4.90 ± 1.29[c]

F1) cow milk; F2) cow milk after skimming and the first thermal treatment; F3) concentrated milk after the second thermal treatment; F4) concentrated infant formula after the last thermal treatment; F5) the infant formula final product; and S) milk whey used as an ingredient.
Data of NPN represents mean ± SD

Differents letters in the same column indicates statistical significant differences at the level of $p<0.001$

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ONLINE-ONLY DATA SUPPLEMENTS:

Table 1S. Milk protein-derived peptides identified in the permeate obtained from raw milk samples (F1) subjected to a hydrolysis process that simulates gastrointestinal digestion.

Detection time (min)	Observed mass (Da)	Protein fragment	Sequence	Activity	References
12.8	460.3	Serum albumin (421-424)	TLVE		
13.5	617.2	αs1-casein (7-10)	VSSS		
13,5-13,8	501,2-501,4	Serum albumin (530-533)	ELLK		
16.7	543.4	Lactoferrin (96-100 y 437-441)	AVVKK		
17	437.1	β-lactoglobulin (100-102) and αs1-casein (102-104)	KKY		
17.3	516.3	κ-casein (111-114)	KKNQ		
17.4	346.2	β-casein (164-169)	LQS	Fragment from LQSW (ACE-inhibitory activity)	Maeno et al., 1996
17.6	543.3	αs1-casein (100-103)	RLKK		
17,6-17,8	445.2	κ-casein (91-94)	QPTT		
18.5	648.3	β-casein (164-169)	SLSQSK	Fragment from PPQSVLLSLSQSKVLPVPE (ACE-inhibitory activity)	Yamamoto et al., 1994

18.5	484.3	κ-casein (74-79)	KPAAV		
18,8-19,0	648.3	Lactoferrin (644-649)	NTECLA		
19	616.4	β-casein (94-99)	GVSKVK		
19.3	616.4	Serum albumin (440-444)	PESER		
19.4	660.3	Lactoferrin (258-263)	RSVDGK		
20,1-20,3	516,2-516,3	β-casein (101-105)	AMAPK	Fragment from VKEAMAPK (antioxidant activity)	Korhonen and Pihlanto-Leppäla, 2007
20,6-20,9	449.2	Lactoferrin (372-376)	ATAST		
21,7-22,2	645.3	β-casein (100-105)	EAMAPK	Fragment from VKEAMAPK (antioxidant activity)	Korhonen and Pihlanto-Leppäla, 2007
22.8	488.3	β-casein (94-98)	GVSKV		
23,4-24,1	674.3	β-casein (1-5)	RELEE	Fragment from RELEELNVPGEIVESLSSSEESITR (immunomodulatory activity)	Coste et al. 1992
23,8-24,0	872.4	β-casein (98-105)	VKEAMAPK	Antioxidant activity	Korhonen and Pihlanto-Leppäla, 2007
24,3-24,4	416.2	β-casein (1-3) and α-	REL		

		lactalbumin(10-12)			
24.9	400,2-400,3	αs1-casein (1-3)	RPK		
24.9	449.3	αs1-casein (151-153)	RQF		
25.1	598.3	β-casein (50-54)	HPFAQ	ACE-inhibitory activity and fragment from IHPFAQTQ (anti-amnestic activity)	Quiros et al., 2005; Asano et al., 1991
25.4	656.4	αs1-casein (99-103)	LRLKK		
25.8	656.4	Lactoferrin (350-355)	VGPEEQ		
27.2	528.4	αs1-casein (136-140)	IGVNQ		
27,4-27,5	528.4	β-casein (25-28)	RINK	Fragment from RELEELNVPGEIVESLSSSEESITRINK (immunomodulatory activity)	Hayes et al., 2007
28	807.3	β-lactoglobulin (62-68)	ENDECAQ		Rahali et al., 2000
28.3	807.4	κ-casein (18-24)	FSDKIAK	Antibacterial and ACE-inhibitory activity	López-Expósito et al., 2006
28.7	685.2	Serum albumin (370-375)	STVFDK		
29.5	635.3	α-lactalbumin (69-74)	SSNICN		
29,5-29,7	803,3-803,4	κ-casein (162-169)	VQVTSTAV		Contreras et al., 2009
29,9-30	780.4	β-casein (169-175)	KVLPVPQ	ACE-inhibitory activity	Maeno et al., 1996

31,1-31,5	612.2	αs1-casein (151-154)	RQFY		
32,3-32,4	501.3	αs2-casein (164-167)	LKKI	fragment from LKKISQ (ACE-inhibitory activity)	López-Expósito et al., 2007
32.7	673.3	β-casein (184-189)	DMPIQA		
33.4	596.3	αs1-casein (150-153)	FRQF	ACE-inhibitory activity	Contreras et al., 2009
33.7	583.3	Lactoferrin (493-498)	PGADPK		
34,8-34,9	400,2-400,3	Lactoferrin (570-572)	RLL		
40.5	786.4	κ-casein (74-79)	LQWQVL		
42.3	786.3	κ-casein (117-123)	TEIPTIN		
43,1-43,2	627,2-627,3	αs1-casein(91-95)	YLGYL	Fragment from RYLGYLE (α-casomorphin with opioid activity)	Clare and Swaisgood, 2000
48.7	462.2	Serum albumin (426-429)	SRSL		
51,3-51,6	675.3	αs1-casein (29-34)	PEVFGK	Fragment from FPEVFGK (ACE-inhibitory activity)	van der Ven et al., 2002
52,8-53	741,3-741,4	Serum albumin (569-575)	VEGPKLV		
57.4	474.1	β-casein (90-93)	PEVM		

Table 2S. Milk protein-derived peptides identified in the permeate obtained from skimmed milk samples (F2) subjected to a hydrolysis process that simulates gastrointestinal digestion.

Detection time (min)	Observed mass (Da)	Protein fragment	Sequence	Activity	References
13.2	501.2	Serum albumin (530-533)	ELLK		
15.1	387.2	Lactoferrin (108-110)	QLQ		
15.8	574.2	Lactoferrin (500-504)	RLCAL		
16.4	502.2	αs2-casein (167-170)	ISQR	Fragment from LKKISQRYQKFALPQY (antibacterial activity)	Recio and Visser, 1999
16.7	543.3	Lactoferrin (96-100 y 437-441)	AVVKK		
17.3	516.3	Serum albumin (520-523)	KQIK	Light antifungal activity	Garibotto et al., 2010
17.6	445.2	Lactoferrin (620-623)	KNGK		
17.9	801.3	β-lactoglobulin (77-83)	KIPAVFK		
18.5	648.3	Lactoferrin (644-649)	NTECLA		
19.0	616.4	Serum albumin (440-444)	PESER		
20.1	516.3	Serum albumin (76-80)	KVASL		

20.5	449.2	Lactoferrin (372-376)	ATAST		
21.7	645.3	β-casein (100-105)	EAMAPK	Fragment from VKEAMAPK (antioxidant activity)	Korhonen and Pihlanto-Leppäla, 2007
22.5	488.1	β-casein (94-98)	GVSKV		
23.7	872.5	β-casein (98-105)	VKEAMAPK	Antioxidant activity	Korhonen and Pihlanto-Leppäla, 2007
25.0	488.1	Serum albumin (248-251)	DLLE		
25.4	656.3	αs1-casein (99-103)	LRLKK		
27.4	528.4	β-casein (25-28)	RINK	Fragment from RELEELNVPGEIVESLSSSEESITRINK (immunomodulatory activity)	Hayes et al., 2007
27.9	963.4	κ-casein (96-103)	ARHPHPHL	Fragment from ARHPHPHLSFM (antioxidant activity)	Korhonen and Pihlanto-Leppäla, 2007
29.6	819.3	αs1-casein (94-104)	LRLKKY		
30.1	779.4	β-lactoglobulin (133-138)	LEKFDK		Ortiz-Chao et al., 2009
32.3	501.2	αs2-casein (164-167)	LKKI	Fragment from LKKISQ (ACE-inhibitory activity)	López-Expósito et al., 2007

33.2	596.3	αs1-casein (150-153)	FRQF	ACE-inhibitory activity	Contreras et al., 2009
33.6	583.3	Serum albumin (383-387)	PQNLI		
38.3	746.3	Lactoferrin (559-564)	DWAKNL		
42.6	786.3	κ-casein (74-79)	LQWQVL		
43.0	627.2	αs1-casein (91-95)	YLGYL	Fragment from RYLGYLE (α-casomorphin with opioid activity)	Clare and Swaisgood, 2000
44.4	834.4	αs2-casein (55-60)	GSSSEE		
51.6	675.3	αs1-casein (29-34)	PEVFGK	Fragment from FPEVFGK (ACE-inhibitory activity)	Recio and Visser, 1999

Table 3S. Milk protein-derived peptides identified in the permeate obtained from concentrated milk samples (F3) subjected to a hydrolysis process that simulates gastrointestinal digestion.

Detection time (min)	**Observed mass (Da)**	**Protein fragment**	**Sequence**	**Activity**	**References**
8.0	468.2	Serum albumin (82-85)	ETYG		
13.3	501.3	Serum albumin (530-533)	ELLK		
17.7	445.2	κ-casein (91-94)	QPTT		
18.7	648.3	β-casein (164-169)	SLSQSK	Fragment from PPQSVLLSLSQSKVLPVPE (ACE-inhibitory activity)	Yamamoto et al., 1994
19.9	376.3	κ-casein (166-169)	STAV	Fragment from VTSTAV (ACE-inhibitory activity)	FitzGerald et al., 2004
20.5	449.2	α-lactalbumin (110-113)	LCSE	Fragment from ALCSEK (antibacterial activity)	Pellegrini et al., 1999
21.7	645.2	β-lactoglobulin (137-142)	DKALKA		
22.2	614.2	αs1-casein (120-124)	LHSMK		
22.6	445.2	Serum albumin (515-518)	LPDT		
23.7	872.4	β-casein (98-105)	VKEAMAPK	Antioxidant activity	Korhonen and Pihlanto-Leppäla,

					2007
24.1	674.2	β-casein (1-5)	RELEE	Fragment from RELEELNVPGEIVESLSSSEESITRINK (immunomodulatory activity)	Coste et al., 1992
25.0	449.2	αs1-casein (151-153)	RQF		
26.2	819.4	β-lactoglobulin (2-8)	IVTQTMK		Corzo- Martínez et al., 2009
26.5	507.3	αs1-casein (90-93)	RYLG	Fragment from RYLGYL (opioid antagonist)	Chiba et al., 1989
32.4	501.3	αs2-casein (164-167)	LKKI	Fragment from LKKISQ (ACE-inhibitory activity)	López-Expósito et al., 2007
32.8	673.3	β-casein (184-189)	DMPIQA	Fragment from RDMPIQAF (ACE-inhibitory activity)	Yamamoto et al., 1994
33.4	596.3	Serum albumin (89-93)	DCCEK		
34.9	400.4	Lactoferrin (570-572)	RLL		
37.3	673.3	β-casein (157-162)	FPPQSV		Hernández-Ledesma et al., 2004
43.3	627.2	αs1-caseína (91-95)	YLGYL	Fragment from RYLGYLE (α-casomorphin with opioid activity)	Clare and Swaisgood ,2000

43.9	544.2	κ-casein (76-79)	WQVL		Nass et al., 2008
73.0	354.3	αs1-caseína (128-130)	HAQ		

Table 4S. Milk protein-derived peptides identified in the permeate obtained from milk serum employed as ingredient for infant formula manufacturing (S) subjected to a hydrolysis process that simulates gastrointestinal digestion.

Detection time (min)	**Observed mass (Da)**	**Protein fragment**	**Sequence**	**Activity**	**References**
7.2	468.1	Serum albumin (82-85)	ETYG		
7.6	468.1	κ-casein (109-112)	PPKK	Fragment from MAIPPKK (antithrombotic activity)	Jolles et al., 1986
9.1	555.1	κ-casein (62-67)	AKPAAV		
18.2	493.2	β-lactoglobulin (24-28)	MAASD	Fragment from WYSLAMAASDI (antioxidant activity)	Pihlanto, 2006
19.0	616.3	β-casein (94-99)	GVSKVK		
19.3	445.2	β-lactoglobulin (125-128)	TPEV		
19.8	376.3	κ-casein (166-169)	STAV	Fragment from VTSTAV (ACE-inhibitory activity)	FitzGerald et al., 2004
20.6	439.3	αs1-casein (150-152)	FRQ	Fragment LFRQ (ACE-inhibitory activity)	Meisel et al., 2006
23.0	633.3	κ-casein (162-167)	VQVTST		Reid et al., 1997

23.3	574.3	κ-casein (159-163)	INTVQ		
25.1	776.3	κ-casein (138-145)	AVESTVAT		
25.7	646.3	β-casein (53-58)	AQTQSL	Fragment from AQTQSLVYP (ACE-inhibitory activity)	Kohmura et al., 1990
28.6	549.3	Serum albumin (407-410)	IVRY		
29.0	514.3	Serum albumin (456-459)	LNRL		
29.5	803.4	κ-casein (162-169)	VQVTSTAV		Contreras et al., 2009
30.1	801.3	β-lactoglobulin (83-89)	KIDALNE		Chicón et al., 2008
30.8	452.2	β-lactoglobulin (20-23)	YSLA	Fragment from WYSLA (antioxidant activity)	Hernández-Ledesma et al., 2007
32.6	643.4	α-lactalbumin (96-101)	LDKVGI		
32.8	537.4	β-lactoglobulin (146-149)	HIRL	β-lactoquinine (ACE-inhibitory activity)	Mullally et al., 1996
34.0	400.3	Lactoferrin (570-572)	RLL		
35.8	675.2	β-lactoglobulin (25-31)	AASDISL		
39.1	805.3	β-casein (155-161)	VMFPPQS		
87.9	356.4	Lactoferrin (29-32)	LGAP		

Table 5S. Milk protein-derived peptides identified in the permeate obtained from concentrated manufactured formual (F4) subjected to a hydrolysis process that simulates gastrointestinal digestion.

Detection time (min)	**Observed mass (Da)**	**Protein fragment**	**Sequence**	**Activity**	**References**
8.2	468.2	Serum albumin (82-85)	ETYG		
8.3	447.3	Lactoferrin (177-180)	GENQ		
13.5	501.3	Serum albumin (94-97)	QEPE		
13.5	501.3	αs1-casein (121-124)	HSMK		
14.3	273.1	Lactoferrin (344-345) and β-lactoglobulin (40-41)	RV		
16.6	502.2	Lactoferrin (428-431)	RPTE		
17.5	437.1	αs1-casein (103-105)	KYK		
17.7	516.2	Serum albumin (541-544)	EQLK		
18.4	661.3	αs1-casein (135-140)	MIGVNK		
18,8-18,9	648.3	β-casein (164-169)	SLSQSK	Fragment from PPQSVLLSLSQSKVLPVPE (ACE-inhibitory activity)	Yamamoto et al., 1994

19.2	470,2-470,3	as1-casein (105-108) and serum albumin (413-417)	KVPQ	Fragment from KKYKVPQ (ACE-inhibitory activity)	Hernández-Ledesma et al. ,2007
19,9-20,0	376,2-376,3	κ-casein (166-169)	STAV	Fragment from VTSTAV (ACE-inhibitory activity)	FitzGerald et al., 2004
20,2-20,3	516.2	Lactoferrin (4-7)	KNVR	Fragment from APRKNVRW (antibacterial activity)	Recio et al., 1998
20.7	449.3	α-lactalbumin (110-113)	LCSE	Fragment from ALCSEK (antibacterial activity)	Pellegrini et al., 1999
20.7	449.3	Serum albumin (10-12)	RFK		
21.8	645.2	Lactoferrin (88-92)	PQTHY	Fragment from AGIYGTKESPQTHYY (immunomodulatory activity)	Hagiwara et al. ,1995
21.9	645.3	β-casein (100-105)	EAMAPK	Frafment from VKEAMAPK (antioxidant activity)	Korhonen and Pihlanto-Leppäla, 2007
22,4-22,5	614.3	αs1-casein (120-24)	LHSMK		
22.7	445.2	Serum albumin (515-518)	LPDT		
23,2-23,3	572,1-572,3	β-lactoglobulin (71-75)	IIAEK	Lactostatin (hipocholesterolemic, inhibitor of cholesterol absorption and ACE-inhibitory activity)	Nagaoka et al., 2001
23.3	572.1	Lactoferrin (146-151)	QGAVAK		

23.9	872.4	β-casein (98-105)	VKEAMAPK	Antioxidant activity	Korhonen and Pihlanto-Leppäla, 2007
24.1	510.1	Serum albumin (565-569)	ACFAV		
24.3	674.3	β-lactoglobulin (78-83)	IPAVFK	Antibacterial activity	Pellegrini et al., 2001
25.2	449.2	αs1-casein (151-153)	RQF		
25.2	604.2	Serum albumin (457-461)	NRLCV		
25.4	488.1	β-casein (205-208)	FPII		
25.5	1336.6	αs1-casein (80-90)	HIQKEDVPSER	Antioxidant activity	Gupta et al., 2010
26,7-26,8	507.3	αs1-casein (90-93)	RYLG	Fragment from RYLGYL (opioid antagonist)	Chiba et al., 1989
27.6	528.4	αs1-casein (99-102)	LRLK		
28.2	469.3	κ-casein (26-29)	IPIQ	Fragment from IPIQYVL (antioxidant activity)	Hernández-Ledesma et al., 2005
29.6	803,3-803,4	κ-casein (162-169)	VQVTSTAV		Contreras et al., 2009
30.6	833.3	β-lactoglobulin (148-154)	RLSFNPT		
31-31,1	452.2	β-lactoglobulin (20-23)	YSLA	Fragment from WYSLAM (antioxidant	Hernández-

				activity)	Ledesma et al., 2007
31.7	398.3	Lactoferrin (591-594)	VAPN		
31.7	398.2	Lactoferrin (409-412)	PVLA		
32.5	501.3	αs2-casein (164-167)	LKKI	Fragment from LKKISQ (ACE-inhibitory activity)	López-Expósito et al., 2007
32.6	501.3	β-casein (27-30)	NKKI		Gómez-Ruiz et al., 2007
32.8	627.3	β-casein (61-66)	PFPGPI	Cathepsin B inhibitor	Lee and Lee, 2000
32.9	673.3	κ-casein (125-131)	IASGEPT		
32.9	673.3	β-casein (177-182)	AVPYPQ	Fragment from AVPYPQR (ACE-inhibitory and antioxidant activity)	
33.7	583.3	Serum albumin (383-387)	PQNLI		
33.8	583.2	Lactoferrin (493-498)	PGADPK		
34,1-35,0	400,3-400,4	Lactoferrin (570-572)	RLL		
37.1	520.2	κ-casein (53-56)	NQFL		
38.4	583.3	β-lactoglobulin (20-24)	YSLAM	Fragment from WYSLAM (antioxidant activity)	Hernández-Ledesma et al., 2007

38.8	6103	Serum albumin (450-454)	DYLSL		
40,2-40,3	680.3	κ-casein (103-108)	LSFMAI		Hernández-Ledesma et al. ,2004
43.4	627,2-627,3	αs1-casein (91-95)	YLGYL	Fragment from RYLGYLE (α-casomorphin with opioid activity)	Clare and Swaisgood, 2000
43.9	544.2	κ-casein (76-79)	WQVL		Nass et al., 2008
46.4	391.3	κ-casein (25-27)	YIP	Fragment from YIPIQYVLSR (immunomodulatory, opiod antagonist and ACE-inhibitory activity)	
53	741,3-741,4	Serum albumin (569-575)	VEGPKLV		
57.4	474.1	β-casein (90-93)	PEVM		
72.2	372.2	Serum albumin (569-575)	EPQ		
72.8	354.3	αs1-casein (128-130)	HAQ		

Table 6S. Milk protein-derived peptides identified in the permeate obtained from final infant formula (F5) subjected to a hydrolysis process that simulates gastrointestinal digestion.

Detection time (min)	**Observed mass (Da)**	**Protein fragment**	**Sequence**	**Activity**	**References**
8	468.2	Serum albumin (82-85)	ETYG		

8,0-8,5	446,2-446,3	κ-casein (137-140)	EAVE		
10.8	399.2	αs1-caseína (1-3)	RPK	ACE-inhibitory activity	Shahidi and Zhong, 2008
13,5-13,7	501,2-501,4	Serum albumin (530-533)	ELLK		
14.3	273.2	Lactoferrin (344-345) and b-lactoglobulin (40-41)	RV	Fragment from RVY (ACE-inhibitory activity)	Cadee and Mallee ,2007
15.5	387.3	Serum albumin (114-116, 273-275, 375-377)	KLK	Fragment from KLKLLLLLKLK with antimicrobial and immunomodulatory activity	Aichinger et al., 2008
16.2	287.1	Serum albumin, Lactoferrin, αs1-casein, αs2-casein and β-lactoglobulin	RL		Mullally et al., 1996
16.5	5933	Lactoferrin (277-281)	KFGKN	Fragment from lactoferrampin (WKLLSKAQEKFGKNKSR) with antibacterial activity	van der Kraan et al,. 2004
16.6	502,2-502,3	Lactoferrin (428-431)	RPTE		
16.8	502.2	αs2-casein (42-45)	EVVR		
17.3	524.3	αs1-casein (78-81)	QKHI		
17.4	524.3	β-casein (59-62)	VYPF	Fragment from VYPFPG (ACE-inhibitory activity)	Abubakar et al., 1998

17.4	623.2	κ-casein (98-102)	HPHPH		
17.6	617.2	αs1-casein (35-39)	EKVNE		
17,6-17,8	445.2	κ-casein (91-94)	QPTT		
18	515.2	Serum albumin (273-276)	KLKE		
18.2	801.3	αs2-casein (183-188)	VYQHQK	Fragment of casocidin-I (antibacterial activity)	Zucht et al., 1995
18.3	661.2	Lactoferrin (200-206)	QDGAGDV		
18.4	661.3	αs2-casein (140-144)	DMEST		
18.7	648.3	Serum albumin (181-185)	IETMR		
18,8-19,0	648.3	Lactoferrin (644-649)	NTECLA		
19.2	470.2	αs1-casein (105-108) and serum albumin (413-417)	KVPQ	Fragment from KKYKVPQ (ACE-inhibitory activity)	Hernández-Ledesma et al., 2007
19,4-19,7	398.1	β-lactoglobulin (78-81)	IPAV	Fragment from IPAVFK (antibacterial activity)	Pellegrini et al., 2001
19,4-20,0	376,2-376,3	κ-casein (166-169)	STAV	Fragment from VTSTAV (ACE-inhibitory activity)	FitzGerald et al., 2004
20,1-20,3	516,2-516,3	β-casein (101-105)	AMAPK	Fragment from VKEAMAPK (antioxidant activity)	Korhonen and Pihlanto-Leppäla, 2007

20,6-20,8	449.2	Lactoferrin (372-376)	ATAST		
20.9	449.4	β-lactoglobulin (78-81)	ECAQ		
21.7	643.3	β-lactoglobulin (78-81)	ENSAEP		
21,7-21,9	645,2-645,3	β-casein (100-105)	EAMAPK	Fragment from VKEAMAPK (antioxidant activity)	Korhonen and Pihlanto-Leppäla, 2007
22.5	445.2	Serum albumin (515-518)	LPDT		
22,4-22,7	614.3	αs1-casein (120-24)	LHSMK		
22.3	572.3	κ-casein (71-75)	AQILQ	Fragment from PAAVRSPAQILQ (antibacterial activity)	López-Expósito et al., 2006
23.2	572.3	Lactoferrin (579-583)	KPVTE		
23,2-23,3	572,1-572,4	β-lactoglobulin (71-75)	IIAEK	Lactostatin (hipocholesterolemic, inhibitor of cholesterol absorption and ACE-inhibitory activity)	Nagaoka et al., 2001
23.3	572.4	Lactoferrin (146-151)	QGAVAK		
23.6	436.3	β-lactoglobulin (40-42)	RVY	ACE-inhibitory activity	Matsufuji et al., 1994
23,9-24,0	872.4	β-casein (98-105)	VKEAMAPK	Antioxidant activity	Korhonen and Pihlanto-Leppäla, 2007

24.1	518.2	αs1-casein (57-60)	IKQM		
24.3	488.2	αs1-casein (151-153)	NENL	Fragmen from VLNENLLR (antibacterial activity)	Hayes et al., 2007
24,3-24,4	674,2-674,3	β-lactoglobulin (78-83)	IPAVFK	Antibacterial activity	Pellegrini et al., 2001
24.7	673.3	κ-casein (117-122)	TEIPTI		
25,2-25,5	449.2	αs1-casein (151-153)	RQF		
25.7	1336.6	αs1-casein (80-90)	HIQKEDVPSER	Antioxidant activity	Gupta et al., 2010
25.9	646.3	Lactoferrin (402-408)	AGKCGLV		
26.3	819.4	Serum albumin (229-235)	FGERALK		
26.3	819.3	Serum albumin (363-369)	DDPHACY		
26,6-26,8	507.3	αs1-casein (90-93)	RYLG	Fragment from RYLGYL (opioid antagonist)	Chiba et al., 1989
27.1	633.3	αs1-casein (104-108)	YQVPQ		
27.3	615.3	α-lactalbumin (54-58)	QINNK	Modulates functions of non classical cadherin	Blaschuk et al. 1999
27.4	615.3	αs1-casein (184-189)	NPIGSE	Fragment from SDIPNPIGSENSEK (antibacterial activity)	Hayes et al., 2007
27.5	528.4	αs1-casein (97-100)	QLLR		

27,4-27,5	528.4	β-casein (25-28)	RINK	Fragment from RELEELNVPGEIVESLSSSEESITRINK (immunomodulating activity)	Hayes et al., 2007
28.4	567.2	β-lactoglobulin (142-146)	ALPMH	Fragment from ALPMHIR (ACE-inhibitory activity)	Mullaly et al., 1997
28,7-28,9	695.2	Serum albumin (370-375)	STVFDK		
29.3	672.3	Serum albumin (77-88)	VASLRE		
29.5	672.3	β-lactoglobulin (8-13)	KGLDIQ		
29,6-29,7	803,3-803,4	κ-casein (162-169)	VQVTSTAV		Contreras et al., 2009
30,0-30,1	779.4	β-lactoglobulin (133-138)	LEKFDK		Ortiz-Chao et al., 2009
30.9	452.2	Serum albumin (485-488)	PCFS		
31-31,1	452.2	β-lactoglobulin (20-23)	YSLA	Fragment from WYSLAM (antioxidant activity)	Hernández-Ledesma et al., 2007
31.6	398.1	β-lactoglobulin (78-81)	IPAV	Fragment from TKIPAV	Hernández-Ledesma et al., 2004
31,7-31,8	584.2	Lactoferrin (144-149)	PLQGAV		

32.1	526.3	β-lactoglobulin (77-81)	KIPAV	Fragment from TKIPAV	Hernández-Ledesma et al., 2004
32.5	501.2	β-casein (27-30)	NKKI		Gómez-Ruiz et al.,
32.9	673.3	Lactoferrin (232-237)	LNNSRA		
32,7-32,9	627,2-627,4	β-lactoglobulin (76-81)	TKIPAV		Hernández-Ledesma et al., 2004
32,9-33,1	673,2-673,3	β-casein (177-182)	AVPYPQ	Fragment from AVPYPQR (antioxidant and ACE-inhibitory activity)	FitzGerald and Murray, 2006
32,8-32,9	627.3	β-casein (61-66)	PFPGPI	Cathepsin B inhibitor	Lee and Lee, 2000
33.3	532.3	Lactoferrin (491-496)	CAPGAD		
33,2-33,5	596.3	αs1-casein (150-153)	FRQF	ACE-inhibitory activity	Contreras et al., 2009
33.7	583.3	Serum albumin (383-387)	PQNLI		
33.8	596.2	Lactoferrin (211-215)	ETTVF		
34,1-35,0	400,2-400,4	Lactoferrin (570-572)	RLL		
34.6	514.2	αs1-casein (91-94)	YLGY	Fragment from RYLGYLE (α-casomorphin with opioid activity)	Clare and Swaisgood, 2000
35.9	583.3	Lactoferrin (478-483)	TGSCAF		

37	596.3	αs1-casein (113-117)	PNSAE		
37.3	759.3	αs1-casein (150-154)	FRQFY		
39.2	805.3	β-lactoglobulin (150-156)	SFNPTQL		Zeece et al., 2008
40.2	680.3	β-lactoglobulin (142-147)	ALPMHI	Fragment from ALPMHIR (ACE-inhibitory activity)	Mullally et al., 1997
40,2-40,3	680.3	κ-casein (103-108)	LSFMAI		Hernández-Ledesma et al., 2004
42,5-42,8	414,2-414,3	αs1-casein (197-200)	PLW	ACE-inhibitory activity	Maruyama et al., 1987
43,3-43,5	627.3	αs1-casein (91-95)	YLGYL	Fragment from RYLGYLE (α-casomorphin with opioid activity)	Clare and Swaisgood, 2000
45.7	688.3	Serum albumin (485-488)	LVNELT		
46.3	391.4	β-casein (190-192)	FLL		
52,8-53	741,3-741,4	Serum albumin (569-575)	VEGPKLV		
73	372.3	αs1-casein (185-188)	PIGS	Fragment from SDIPNPIGSENSEK (antibacterial activity)	Hayes et al., 2006

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